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The Putative Apoptosis Inhibitor IEX-1L Is a Mutant Nonspliced Variant of p22$^{PRG1/IEX-1}$ and Is Not Expressed *in Vivo*

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IEX-1L has been claimed to act as an apoptosis inhibitor involved in NFκB-mediated survival in Jurkat cells [Wu *et al.* (1998) *Science* 281, 998–1001]. It represents a mutant nonspliced variant of the early response gene p22$^{PRG1/IEX-1}$ exhibiting one insertion and two deletions compared to the genomic sequence of p22$^{PRG1/IEX-1}$. Direct DNA sequencing of PCR products generated from human genomic DNA only detected the regular genomic sequence of p22$^{PRG1/IEX-1}$. No IEX-1L mRNA could be identified by RT-PCR analysis and subsequent DNA sequencing of total, nuclear, or cytoplasmic RNA fractions from PMA-stimulated Jurkat cells. The only functional transcript residing in the cytoplasm is regularly spliced p22$^{IEX-1/PRG1}$ mRNA. Substantial amounts of nonmutated nonspliced p22$^{IEX-1/PRG1}$ pre-mRNA were identified in the nucleus. Thus, IEX-1L seems to be a mutant variant of p22$^{IEX-1/PRG1}$ not existing *in vivo*. Antiapoptotic effects obviously represent transdominant negative inhibition of endogenous p22$^{PRG1/IEX-1}$ in Jurkat cells and several other tumor cell lines. © 1999 Academic Press

In search of NFκB dependent genes that confer resistance to TNFα-mediated cell death [1–4], Wu and colleagues recently described a putative NFκB responsive gene—IEX-1L—claimed to represent an apoptosis inhibitor expressed in Jurkat cells as well as in other cell lines [5]. IEX-1L is a mutant nonspliced variant of the early response gene p22$^{IEX-1/PRG1}$. Compared to all other published cDNA and genomic sequences of p22$^{IEX-1/PRG1}$ [6–8] the nucleotide sequence of IEX-1L exhibits one single base insertion and two single base deletions that account for a nonspliced transcript retaining the reading frame for native p22$^{IEX-1/PRG1}$ including an insert of 37 amino acids representing the intron [5]. p22$^{IEX-1/PRG1}$ is widely expressed in humans, rats and mice and its expression is associated with the induction of proliferation [9–11], cellular adaptation [7, 8] and stress response [6, 11]. Moreover, p22$^{IEX-1/PRG1}$ represents a p53-responsive gene that might be involved in p53-dependent growth control upon DNA damage or other insults [12, 13] Recent promoter studies also revealed functional binding sites for NFκB [13]. However, the exact function of p22$^{IEX-1/PRG1}$ remained unclear until Wu *et al.* reported that overexpression of IEX-1L mediates resistance to TNFα as well as Fas dependent cell death in various cellular systems [5]. Since IEX-1L cannot be regarded as a simple splicing variant of p22$^{IEX-1/PRG1}$ we hypothesize that a second closely related gene may encode IEX-1L or that an artificially generated mutant variant of p22$^{IEX-1/PRG1}$ has been cloned. In this study, we investigated the relation of p22$^{IEX-1/PRG1}$ to its putative nonspliced mutant variant IEX-1L in the context of TNFα- or PMA-dependent activation of NFκB.

MATERIALS AND METHODS

Materials. Oligonucleotides were custom synthesized by Biometra (Göttingen, FRG), and cell culture media were from Biochrom/Seromed (Hamburg, FRG).

Cell culture. Jurkat cells were kept in culture using RPMI supplemented with 1% glutamine and 5% FCS. 818-4 and PT45-1 pancreatic carcinoma cells as well as HeLa cells were cultured in RPMI (1% glutamine; 10% FCS) and CHO cells in HAMS F12 (1% glutamine; 5% FCS).

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Abbreviations used: IEX-1L, immediate early gene on X-radiation-1 long; PRG1, PACAP response gene 1; PMA, phorbol myristoyl acetate; FCS, fetal calf serum; TNFα, tumor necrosis factor α.

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PCR on genomic DNA and direct DNA sequencing. Several independent samples of human DNA (200 ng) were used for PCR with various sets of primers targeted to exon 1 and exon 2 as well as to the intron of $p22^{IEX-1/PRG1}$: 5′-ctccgctc ggctcaccat-3′ (forward-1, positions 5–22); 5′-agtgcggggagtcacagttagaag-3′ (reverse, positions 621–598); 5′-gtgagtatcgccgaagtg-3′ (forward-2, positions 231–248). PCR conditions were 5 min 95°C; 2 min 95°C, 1 min 60°C, 1 min 72°C for 28 cycles; 72°C 10 min. PCR products were analyzed by PAGE and subsequent EtBr staining. For direct DNA-sequencing, PCR products were separated by agarose gel electrophoresis, bands excised and eluted with Qiaspin gel extraction kit (Qiagen, Hilden, FRG). Eluted DNA was submitted to cycle sequencing using the Big-Dye Sequencing Kit (Perkin–Elmer Biosystems, Weiterstadt, FRG) and the PCR primers used above. Sequencing was carried out on an automated DNA sequencer (Abi-Prism 310A, Perkin–Elmer Biosystems, Weiterstadt, FRG).

Preparation of total RNA. Cells (5×10^4) were harvested, washed twice with PBS and submitted to preparation of total RNA using Qiashredder and RNeasy cartridges (Qiagen, Hilden, FRG) according to the manufacturers protocol. For the isolation of separated RNA fractions from cytoplasm and nuclei, cells were lysed in 300 μl of 50 mM Tris/HCl, pH 8.0, 140 mM NaCl, 1.5 mM $MgCl_2$, 0.5% NP-40, 1000 U/ml RNAsin (Promega; Heidelberg, FRG), 1 mM DTT and were incubated for 5 min on ice. Thereafter nuclei were sedimented at 300*g*, 4°C for 2 min and lysed and submitted to RNA preparation as described above. Supernatants were mixed with 900 μl of the Qiashredder lysis buffer and 650 μl of ethanol and loaded to the Rneasy columns. All RNA specimens were treated with DNAseI for 20 min at 37°C and repurified on an RNeasy cartridge.

RT-PCR analysis and indirect sequencing of RT-PCR products. Nuclear, cytoplasmic or total cellular RNA (0.5 μg) from TNFα, PMA-stimulated or unstimulated cell lines were submitted to reverse transcription (*Ready to go* kit, Pharmacia, Freiburg, FRG) using dT_{15} primers. Two microliters of the RT-mixture was directly submitted to PCR (30 μl) using the same primers targeted to the exons and the intron of $p22^{PRG1/IEX-1}$/IEX-1L, as described above. For detection of the intronless $p22^{PRG1/IEX-1}$ mRNA the cycle condition were: 2 min 95°C; 1 min 95°C, 30 s 60°C, 30 s 72°C for 20–22 cycles; 72°C 10 min. For detection of unspliced $p22^{PRG1/IEX-1}$ the PCR was carried out at 2 min 95°C; 1 min 95°C, 30 s 60°C, 30 s 72°C for 32 cycles; 72°C 10 min. In addition, c-fos mRNA was amplified using commercial PCR-primers (Amplimer, Clontech) and c-fos pre-mRNA was amplified using an exon 1-targeted (5′-aaccgccacgatgatgttctc-3′; forward) and an intron 1-targeted (5′-cctactcatctactggagcgt-3′, reversed) primer. PCR conditions were: 2 min 95°C; 1 min 95°C, 30 s 60°C, 30 s 72°C for 22 or 30 cycles; 72°C 10 min. For control, β-actin was amplified using commercial primers (Amplimer, Clontech; Heidelberg, FRG) RT-PCR products were analyzed by PAGE and EtBr staining. For indirect DNA sequencing, RT-PCR products were ligated into the pCR2.1 TA-cloning vector (Invitrogen, De Schelp, The Netherlands) and transformed into *E. coli* cells (INVαF). Plasmids were isolated (QIAGEN Plasmid Mini Kit, Qiagen, Hilden, FRG) from appropriate colonies upon overnight culture (LB medium plus 80 μg/ml ampicillin). Using IR-labeled (−49) and (−43) universal sequencing primers (MWG-Biotech, Ebersberg, FRG), plasmids were submitted to cycle sequencing and subsequent sequence analysis on an automated DNA sequencer (LICOR 4000L).

Transfection of CHO cells. Using appropriate PCR primers—5′-ctccgctcggcttaccat-3′ (forward) and 5′-gtcagtccagttagaagg-3′ (reverse), a PCR-product containing the entire coding region of $p22^{PRG1/IEX-1}$ was generated by RT-PCR and cloned into a pCMV expression vector (pCR3.1, Invitrogen). In a similar fashion, the unspliced cDNA of $p22^{PRG1/IEX-1}$ (IEX-1L) was generated including the three frameshift mutations (two deletions and one insertion) incorporated by PCR-based *in vitro* mutagenesis. All expression constructs were checked by DNA sequencing using an automated sequence analyzer (LICOR 4000). For transient transfection, CHO-cells raised in HAMS medium containing 5% FCS were plated onto 6-well dishes until 40–50% confluency. After 2 h serum starvation, cells were submitted to lipofection (15 μl/well Lipofectamine, GIBCO-BRL) using 0.5 or 0.2 μg of pCMV-$p22^{PRG1/IEX-1}$, 1.5 or 1.8 μg of pCMV-IEX-1L either alone (total amount was adjusted to 2 μg using pCMVlacZ) or in combination. After a 6 h period at 37°C, 2 volumes of HAMS medium plus 1.5% FCS were added and incubation continued for 16 h at 37°C. Afterward, medium was replaced by HAMS ± FCS. Transfection efficacy was checked by determination of lacZ expression using a commercial Gal ELISA (Boehringer) as well as an *in situ* staining protocol.

Propidium iodide staining and FACS analysis. Transfected CHO cells were incubated (24 and 48 h) in the absence or presence of serum (1.5% FCS). Then, cells were carefully dislodged by gentle trypsinization and washed twice in cold PBS containing 5 mM EDTA. After resuspending cells in 500 μl PBS (+5 mM EDTA), 500 μl chilled EtOH was added dropwise and the mixture was incubated at room temperature for 30 min. Fixed cells were collected by centrifugation, resuspended in 500 μl PBS (+5 mM EDTA), incubated with 20 μg RNaseA (20 μl from 1 mg/ml stock solution) for 30 min at room temperature and subsequently stained with propidium iodide (PI) by adding 500 μl of a 200 μg/ml PI-stock solution. Samples were stored at 4°C in the dark until FACS analysis using a Becton–Dickinson instrument. Sub-G0/G1, G0/G1, and S/G2-M fractions were counted independently and data were expressed as percentage of overall cell count.

Western blot analysis. Cells were lysed in 2× SDS–PAGE sample buffer, heated (95°C) for 5 min and submitted to 12.5% SDS–PAGE. Separated proteins were electroblotted onto PVDF membrane and blots were blocked overnight with 5% nonfat milk powder, 0.1% Tween 20 in PBS (blocking solution) at 4°C. Afterward, blots were exposed for 30 min at room temperature to a rabbit antiserum against a synthetic $p22^{PRG1/IEX-1}$-$peptide^{[67–85]}$ (1000-fold diluted in blocking solution). After extensive washing with blocking solution, blots were exposed to the appropriate second antibody and developed using the Phototope immunoblot detection kit (New England Biolabs).

RESULTS AND DISCUSSION

The nucleotide sequence of IEX-1L deviates in three crucial positions from the genomic $p22^{IEX-1/PRG1}$ sequence. In their paper, Wu *et al.* did not describe the origin of nonspliced IEX-1L mRNA. Comparison of the cDNA sequence of IEX-1L [5] as well as its genomic structure submitted in the GenBank data base with those nucleotide sequences previously reported by several independent groups [6–8, 13, 14] revealed identical nucleotide sequences of both exons, the intron and the promoter region except for three substantial alterations which reside in the sequence of IEX-1L (Fig. 1). At the junction of the 112 bp spanning intron and exon II (ttag-gtcc) one guanine nucleotide (position 344) is deleted (ttag-tcc) causing a lack of an acceptor site for splicing. This alone would result in an in-frame insertion of 37 amino acids (from 111 bp). Also in contrast to all other reported sequences of $p22^{IEX-1/PRG1}$, in exon I of IEX-1L an additional cytosine nucleotide is incorporated at position 215 that per se leads to a frame shift (+1) accounting for translational termination at position 326 of the regularly spliced variant of $p22^{IEX-1/PRG1}$ and at position 431 of the nonspliced IEX-1L. Due to another deletion of a guanine nucleotide within the intron sequence at position 303 that contrasts the

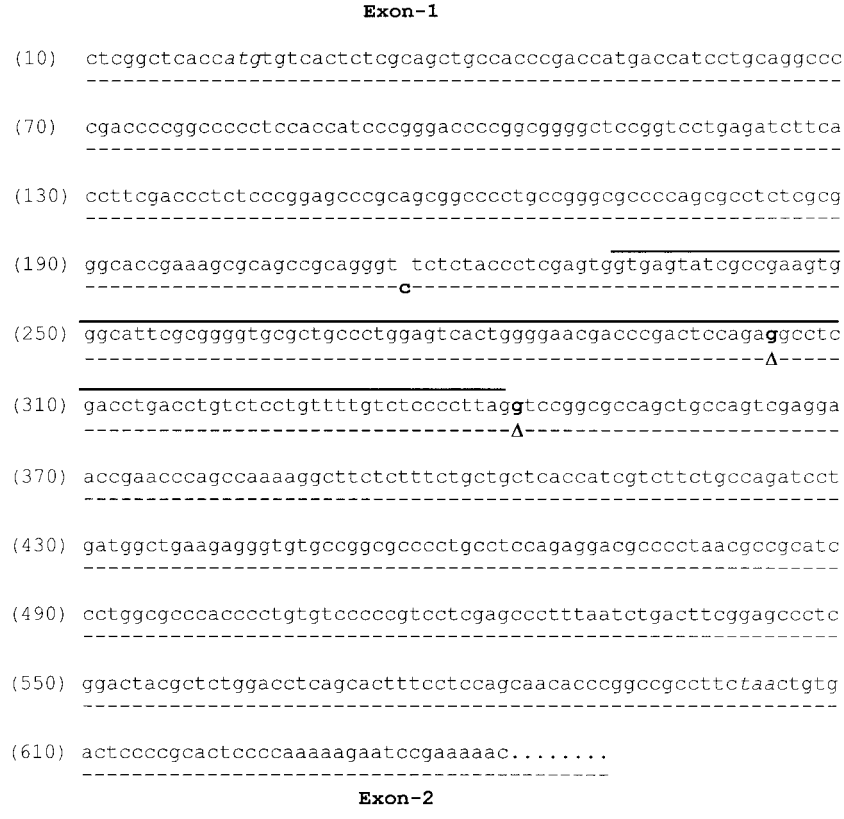

FIG. 1. Comparison of the IEX-1L cDNA sequence with the p22PRG1/IEX-1 consensus sequence. The consensus sequences is derived from five independently reported cDNA- or genomic DNA sequences (GenBank or EMBL Acc. Nos. AF083421, S81914, X96438, Y14551, and Y16737) and is depicted in the upper line. Alignment of the cDNA sequence of IEX-1L (GenBank Acc. Nos. AF071596 and AF039067) is shown below the consensus sequence. Residues matching the consensus sequence are indicated by a horizontal bar, the C-insertion in position 215 is shown in bold and deletions are indicated by Δ. The solid line above the consensus sequence denotes the 112-bp intron sequence. The start and stop codons are depicted in italics.

two other published genomic sequences of $p22^{IEX\text{-}1/PRG1}$, the reading frame is regained for the nonspliced IEX-1L.

No evidence for a second closely related gene encoding IEX-1L. The above-mentioned structural alterations are hardly the result of an unusual splicing mechanism but could reflect the existence of a second gene, although the high degree of sequence conservation, even in the promoter region [13, 14] and the 3′ UTR [6, 7], is rather unusual. To elucidate whether or not a second gene exists, direct sequencing of PCR products from various specimens of human genomic DNA was carried out. When deriving from two genes, these PCR products would start with identical sequences, but beyond position 214 two sequences should emerge. However, all sequencing efforts on several PCR products generated with various sets of primers recognizing identical parts of IEX-1L and $p22^{IEX\text{-}1/PRG1}$ consistently brought about a single sequence with no shifts compatible with the existence of IEX-1L on the genomic level (Fig. 2). This finding rules out the existence of a second $p22^{IEX\text{-}1/PRG1}$ related gene in the human genome.

IEX-1L is not expressed in various PMA- and TNFα-treated cell lines. Wu *et al.* reported that IEX-1L is transcriptionally induced by PMA or TNFα in Jurkat, HeLa, U937, and several other cell lines [5]. Under these conditions, Wu and colleagues detected nonspliced $p22^{IEX\text{-}1/PRG1}$ mRNA by Northern hybridization using a DNA fragment encompassing the intron of $p22^{IEX\text{-}1/PRG1}$ as probe. They detected a band of slightly higher molecular weight compared to regularly spliced $p22^{IEX\text{-}1/PRG1}$ and regarded this as proof for the existence of IEX-1L expression *in vivo.* To verify these observations, we performed RT-PCR on various DNAse treated RNA preparations using exon-targeted or intron-targeted primers followed by direct and indirect DNA sequencing of the obtained PCR products. When analyzing total RNA from Jurkat or PT45-1 pancreatic carcinoma cells upon stimulation with TNFα (Fig. 3), a condition reported to induce $p22^{IEX\text{-}1/PRG1}$ [6, 11, 13, 15], RT-PCR with exon-targeted primers amplified a stimulus related PCR product deriving from the regularly spliced $p22^{IEX\text{-}1/PRG1}$ transcript without the C-insertion in exon 1. In addition to this predominant PCR-product, RT-PCR with exon-targeted primers produced

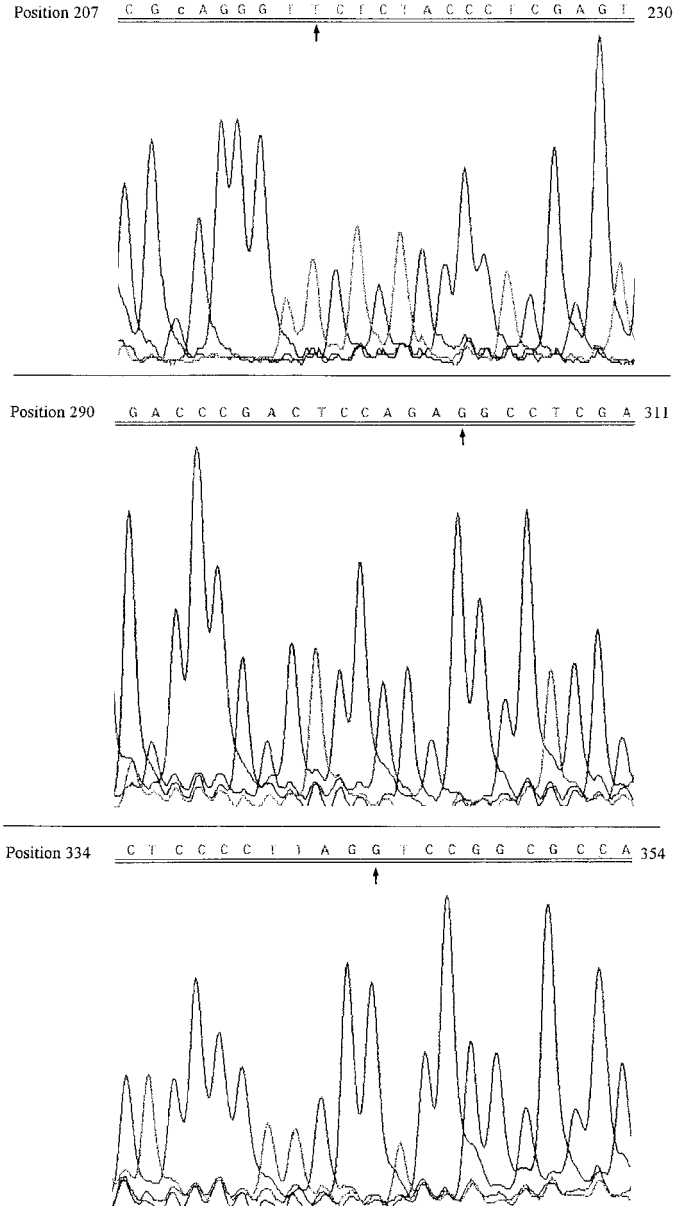

FIG. 2. DNA-sequence of PCR products derived from genomic p22$^{PRG1/IEX-1}$/IEX1-L. Using exon- as well as intron-targeted primers for p22$^{PRG1/IEX-1}$/IEX-1L, PCR products were generated from human genomic DNA. Direct sequencing of these PCR products revealed a single DNA sequence matching the consensus sequence of p22$^{PRG1/IEX-1}$. A representative sequencing result shows those regions containing the three sequence deviations reported for IEX-1L (position 215, CT instead of TC; position 304, GC instead GG and position 344, TC instead GT; the arrows indicate these positions).

a faint DNA fragment exhibiting an extended length of approximately 100 bp. This PCR product comprises the native genomic sequence of p22$^{IEX-1/PRG1}$ including the 112-bp intron, but lacks all three nucleotide alterations reported for IEX-1L (Table 1). RT-PCR with intron specific primers produced also a stimulus dependent PCR-product (Fig. 3) that exhibits again the native sequence of genomic p22$^{IEX-1/PRG1}$ (Table 1). Similar results were obtained for HeLa and 818-4 or Panc-Tu1 pancreatic carcinoma cells (Table 1).

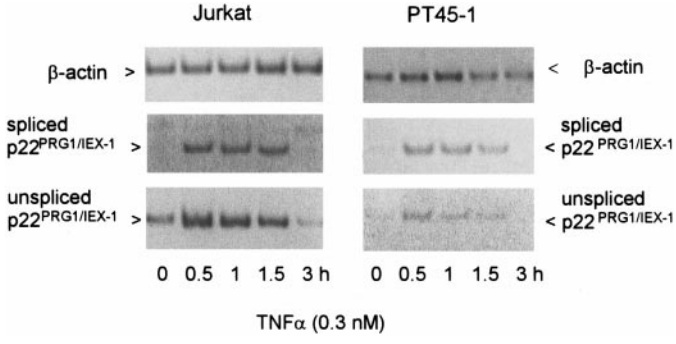

FIG. 3. Presence of spliced or unspliced p22$^{PRG1/IEX-1}$ mRNA in TNFα treated Jurkat and PT45-1 cells. Jurkat or PT45-1 cells were treated with 0.3 nM TNFα for the indicated periods. DNAse treated total RNA preparations were submitted to reverse transcription using oligo-T primers and M-MLV reverse transcriptase. Then, PCR was carried out for: β-actin as internal control (Amplimer primers, Clontech); for spliced or unspliced p22$^{PRG1/IEX-1}$ using exon 1- and exon 2-targeted primers (positions 5 and 621); for unspliced p22$^{PRG1/IEX-1}$ using an intron 1- and the exon 2-targeted primer (positions 231 and 621). A representative EtBr-stained PAA-gel is shown (from three independent experiments).

IEX-1L mRNA detected in PMA stimulated Jurkat cells represents nonspliced pre-mRNA of p22$^{IEX-1/PRG1}$. To check whether the nonspliced p22$^{IEX-1/PRG1}$ mRNA identified in stimulated Jurkat cells represents pre-mRNA we conducted RT-PCR and subsequent DNA sequence analysis on separated preparations of cytoplasmic and nuclear RNA upon treatment with DNAse. Hereby, it was shown that the intron-less PCR product of p22$^{IEX-1/PRG1}$ is detectable mainly in the cytoplasmic but also in the nuclear fraction of PMA-stimulated Jurkat cells (Fig. 4A). In contrast, the intron-containing

TABLE 1

Sequence of Nonspliced p22$^{PRG1/IEX-1}$ in Those Three Relevant Positions Distinct between p22$^{PRG1/IEX-1}$ and IEX-1L

	Nonspliced p22$^{PRG1/IEX-1}$		
Cell line	Position 212–216	Position 302–305	Position 342–345
Jurkat[a]	GTTC	GAGG	AGGT
Jurkat[b]	GTTC	GAGG	AGGT
HeLa[b]	GTTC	GAGG	AGGT
818-4[b]	GTTC	GAGG	AGGT
PT45-1[b]	GTTC	GAGG	AGGT
Panc-Tul[b]	GTTC	GAGG	AGGT
IEX-1L	GTCT	GAGΔ^{G}C	AGΔ^{G}TC

Note. PCR products were generated by RT-PCR on total RNA from [a]PMA- or [b]TNFα-stimulated cell lines. Upon cloning into the pCR2.1 TA-cloning vector (Invitrogen), 24 independent clones were sequenced using an automated DNA sequencer (LICOR 4000L).

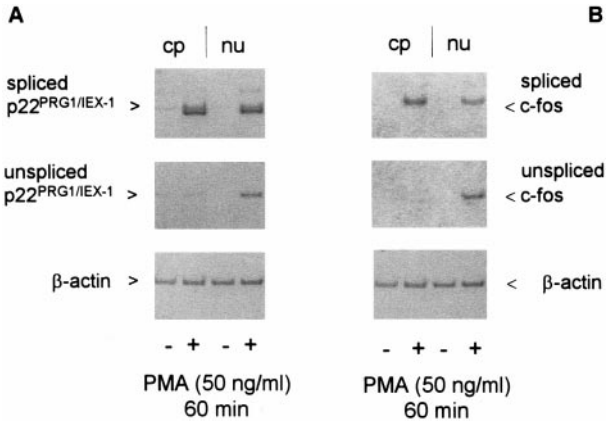

FIG. 4. RT-PCR analysis on cytoplasmic and nuclear RNA for expression of spliced or unspliced $p22^{PRG1/IEX-1}$. Jurkat cells were treated for 1 h with 50 ng/ml PMA or without, then RNA was isolated from nuclear and cytoplasmic fractions followed by DNAse1 digestion. Upon oligo-T primed first strand cDNA synthesis using M-MLV reverse transcriptase, PCR was carried out for (A and B) β-actin as internal control using an amplimer set purchased from Clontech; (a) for spliced or unspliced $p22^{PRG1/IEX-1}$ using exon 1- and exon 2-targeted primers flanking the intron (positions 5 to 621); for unspliced $p22^{PRG1/IEX-1}$ using an intron- and an exon 2-targeted primer (positions 231 to 621); (B) for spliced c-fos using an amplimer set purchased from Clontech; for unspliced c-fos using an exon 1-targeted and an intron 1-targeted primer. A representative EtBr-stained PAA-gel is shown (from four independent experiments); cp, cytoplasmic RNA; nu, nuclear RNA.

PCR product was not detectable in the cytoplasmic fraction of either stimulated or nonstimulated cells, whereas a strong RT-PCR signal came up from nuclear RNA of stimulated cells. A similar pattern was observed when RT-PCR analysis was performed with primers for c-fos (Fig. 4B). Again the intronless PCR product was predominantly present in the cytoplasmic fraction of PMA treated cells, whereas the intron-containing product derived from pre-mRNA was not detectable in the cytoplasm. Conversely, a strong signal was observed for c-fos pre-mRNA in the nucleus of stimulated cells, that also contained mature c-fos mRNA.

These experiments on separated RNA specimens clearly demonstrate that PMA induces the expression of nonspliced pre-mRNA of regular $p22^{IEX-1/PRG1}$ which predominates in the nucleus but does not induce the expression of IEX-1L. Analysis of IEX-1L expression by RT-PCR with intron specific primers or by Northern blotting with an intron-targeted probe, as it has been done by Wu *et al.* [5], produces a signal that could be misinterpreted if no discrimination between mRNA and pre-mRNA is done. Therefore, the appearance of the Northern signal for IEX-1L is due to significant amounts of $p22^{IEX-1/PRG1}$-pre-mRNA exhibiting induction kinetics in parallel to the mature $p22^{IEX-1/PRG1}$-mRNA.

IEX-1L is an artificially mutated variant of $p22^{IEX-1/PRG1}$ acting as a transdominant negative inhibitor. To investigate whether ectopically expressed IEX-1L functionally interferes with $p22^{PRG1/IEX-1}$ we used CHO cells for heterologous expression of these two genes. Upon transfection with two amounts (0.2 and 0.5 μg, adjusted to 2 μg with pCMVlacZ) of an expression vector (pCMV) encoding His-tagged or untagged $p22^{PRG1/IEX-1}$, a dose-dependent increase of $p22^{PRG1/IEX-1}$ protein could be detected in these transfectants. As shown by Western blotting using an antiserum against $p22^{PRG1/IEX-1}$ (Fig. 5), a strong signal came up after 24 h corresponding to a 22-kDa protein. Upon transfection (1.8 and 1.5 μg, adjusted to 2 μg with pCMVlacZ) of IEX-1L generated by PCR-based in-vitro mutagenesis, a more weakly immunolabeled protein was detected exhibiting a slightly higher molecular size (approximately 4 kDa). The reduced labeling may be attributed to a partial loss of immunoreactivity against the $p22^{PRG1/IEX-1}$ antiserum due to the altered protein sequence in the center part of the IEX-1L molecule. By contrast, no signal was observed in pCMVlacZ transfected cells. Cotransfection of IEX-1L and $p22^{PRG1/IEX-1}$ in two different ratios (1.8 μg vs 0.2 μg and 1.5 μg vs 0.5 μg) brought about distinct expression levels compared to cells cotransfected with lacZ. In the presence of IEX-1L the level of $p22^{PRG1/IEX-1}$ is slightly reduced in comparison to its amount in cells cotransfected with lacZ. Conversely, compared to lacZ cotransfection the amount of IEX-1L is much higher when expressed together with $p22^{PRG1/IEX-1}$.

The effect of ectopic expression of $p22^{PRG1/IEX-1}$ on cellular viability of transfected CHO cells was analyzed by propidium iodide staining and subsequent FACS analysis. As summarized in Fig. 6, compared to control transfectants receiving pCMVlacZ (less than 25% apop-

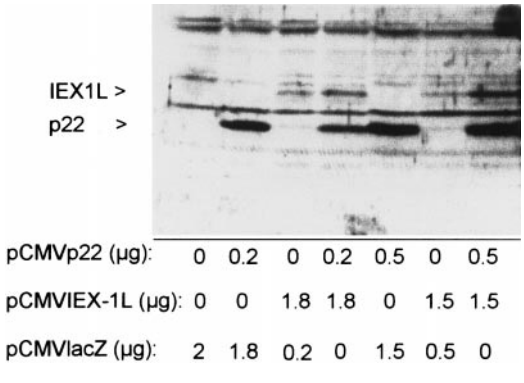

FIG. 5. Ectopic coexpression of $p22^{PRG1/IEX-1}$ and artificial IEX-1L in CHO cells. CHO cells were transfected with various amounts of pCMV expression vectors for $p22^{PRG1/IEX-1}$ or artificial IEX-1L alone, or cotransfected with both. For control, and to adjust the plasmid amount up to 2 μg, pCMVlacZ was used. Upon transfection and serum-starvation for 24 h, cells were lysed in reducing SDS–PAGE sample buffer and lysates were submitted to western blotting.

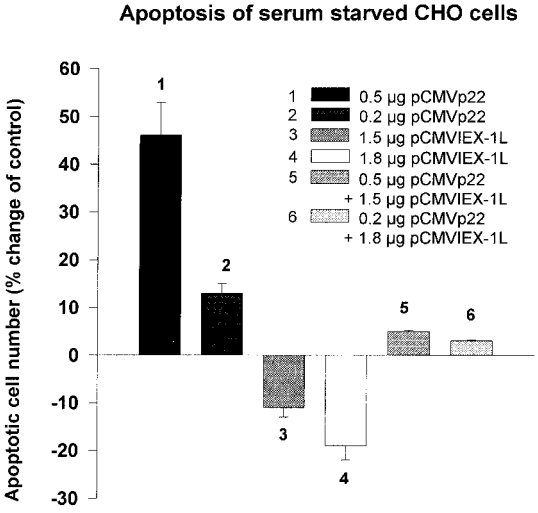

FIG. 6. Altered viability of transfected CHO cells during serum starvation. Upon transfection (total amount of plasmid adjusted to 2 μg with pCMVlacZ) and incubation for 48 h under serum withdrawal, CHO cells were harvested, fixed and stained with propidium iodide. Then, PI-stained cells were counted by FACS analysis using gates for sub-G1, G1, and S/G2-M fractions. The amount of dying cells (sub-G1 fraction) was calculated in percent of total cell count. Data are expressed as percentage of apoptotic cells above or below the basal level (pCMVlacZ alone) and show the mean ± SD of four independent experiments performed in duplicate.

tosis), $p22^{PRG1/IEX-1}$ transfected CHO cells exhibited a strong increase in apoptotic cell number (sub G0/G1 fraction) upon 24 h serum withdrawal. This effect was more pronounced (46 and 13%, respectively) if more plasmid encoding $p22^{PRG1/IEX-1}$ was transfected into CHO cells (0.5 and 0.2 μg, respectively). In contrast, CHO cells transfected with the artificial IEX-1L exhibited a reduced loss of viability upon serum starvation compared to control transfectants. Moreover, the increase in apoptosis observed in $p22^{PRG1/IEX-1}$ transfected CHO cells was almost completely reduced in the presence of IEX-1L, as shown by cotransfection experiments. A nine-fold excess of IEX-1L over $p22^{PRG1/IEX-1}$ expression vectors completely blocked the enhancing effect of $p22^{PRG1/IEX-1}$ on cell death of serum-starved CHO cells.

These findings indicate that the mutant nonspliced variant of $p22^{PRG1/IEX-1}$ apparently acts as a transdominant negative inhibitor of $p22^{PRG1/IEX-1}$. This inhibition may include an altered protein turn over as indicated by the inversed relation of protein levels in cotransfectants. In the absence of serum, overexpression of $p22^{PRG1/IEX-1}$ obviously triggers cell death, a scenario that may be due to a signal conflict [16–18] coming up when pro-proliferative genes are expressed during growth inappropriate conditions, that is i.e., lack of additional surviving factors or presence of proapoptotic factors. In support of this, no apoptosis inducing effect was observed in the presence of serum, but a growth acceleration was noted (result not shown) if CHO cells were transfected with $p22^{PRG1/IEX-1}$, an observation also made by others [11].

As outlined above, we could provide strong evidence that IEX-1L does not exist *in vivo*. The findings that no second closely related gene was identified and no expression of IEX-1L mRNA was detected strongly support the hypothesis that IEX-1L is an artificially mutated variant of $p22^{IEX-1/PRG1}$. Since the source of the cDNA used for differential display and IEX-1L cloning was not reported [5] it can be speculated that during isolation of IEX-1L cDNA three frameshift mutations were incorporated generating a nonspliced form of $p22^{IEX-1/PRG1}$. Theoretically, this triple-mutated variant may be expressed in a distinct tumor cell line which was not checked by our screening program. However, overexpression of this mutant nonspliced form of $p22^{IEX-1/PRG1}$ may indeed inhibit apoptosis, but this would reflect interference as a transdominant negative inhibitor with the as yet not fully defined function of $p22^{IEX-1/PRG1}$.

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